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Award Number: W81XWH-06-1-0510

TITLE: Identifying ECM Mediators of Tumor Cell Dormancy

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REPORT DATE: May 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-05-2007		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 May 2006 – 30 Apr 2007	
4. TITLE AND SUBTITLE Identifying ECM Mediators of Tumor Cell Dormancy				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0510	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Pepper Schedin, Ph.D. E-Mail: pepper.schedin@uchsc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Colorado Health Science Center Aurora, Colorado 80045-0508				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Purpose: Characterize the compositional and functional changes in mammary stroma that result from tamoxifen treatment. Approach: R75 mature female Sprague Dawley rats were randomized into three groups of 25 each; Gp1 nulliparous control, and Gp 2 tamoxifen treated (0.5 mg/tamoxifen body weight, s.c. injection for 30 days) and Gp 3 tamoxifen treated (1.0 mg tamoxifen dose). ECM was harvested from the mammary glands of Gps 1 biochemical and functional characterizations. The ECM preparations have been subjected to LCMS and MALDI-TOF mass spec. Due to technical difficulties also developed two in vitro models to investigate the effects of tamoxifen on mammary stroma. ECM deposited by primary mammary fibroblasts isolated from tamoxifen treated rats, or primary control fibroblasts treated with tamoxifen in culture has been utilized for ECM proteomics method development. Conditions demonstrate fibronectin (FN) is downregulated by tamoxifen, in vitro and in vivo; observations consistent with data demonstrating that FN is up during MEC proliferation and down regulated at times of MEC loss; suggesting that loss of FN may be integral to a tumor suppressive microenvironment investigate functional changes in ECM, MDA-MB-231 cells were pre-mixed with control matrix or matrix isolated from tamoxifen treated rats and orthotically injected into nude mice. Tumor growth was reduced in mice injected with ECM isolated from tamoxifen treated rats, demonstrating that tamoxifen altered EC manner consistent with tumor cell dormancy. Finally, this work identifies ECM mediators of tumor cell dormancy and progression that can be targeted for drug development.					
15. SUBJECT TERMS Tumor microenvironment, epithelia cell –ECM interactions, tumor cell dormancy					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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Table of Contents

	<u>Page</u>
Introduction.....	3
Body.....	3
Key Research Accomplishments.....	12
Reportable Outcomes.....	12
Conclusion.....	13
References.....	13
Appendices.....	NA

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Early stage cancers have long been considered to be less aggressive than late stage cancers because they have accumulated fewer mutations required for metastasis. For breast cancer, recent gene-expression profiling data have challenged this paradigm by identifying very early stage cancers with gene expression profiles similar to that detected in fully metastatic cancers. In fact, numerous studies have demonstrated that having genetic mutations required for metastasis is not sufficient to guarantee a successful metastatic event. These studies indicate that the interaction of the tumor cell with its microenvironment is as rate-limiting for tumor cell progression as is the mutational genotype of the tumor cell. Thus, identifying stromal proteins that determine whether a tumor cell will remain dormant or progress is critical for the clinical control of breast cancer. We propose that the functional unit of cancer is the cancer cell plus its microenvironment, and that both compartments comprise the response to interventions that reduce risk for cancer progression. Specifically, we hypothesize that stromal changes actively contribute to the protection afforded by parity and tamoxifen, two strategies proven to reduce breast cancer risk in women. In this study, we will investigate this hypothesis by characterizing the compositional and functional changes in mammary stroma that result from parity and tamoxifen treatment. To this end we proposed to determine whether breast cancer preventive strategies alter the breast environment, resulting in mammary extracellular matrix (ECM) that suppresses metastatic attributes of tumor cells. Parity and tamoxifen induced changes in mammary ECM composition will be determined by proteomics and changes in matrix function will be determined by *in vitro* assays for metastasis. Mammary ECM proteins elevated by tamoxifen and parity will be candidates for mediators of tumor suppression, whereas ECM proteins decreased by tamoxifen and parity will be candidates for mediators of tumor progression. Further, we will extend these studies to determine whether tumor cell-stroma interactions *in vivo* are altered by tamoxifen and pregnancy. Using a xenograft model, tumor growth and ability of tumor cells to induce a host desmoplastic reaction will be evaluated for tumor cells injected into mammary fat pads of nulliparous, parous and tamoxifen treated mice.

BODY OF REPORT: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work.

Research Accomplishments Associated with Each Task Outlined in the Approved Statement of Work:

Task 1-Animal husbandry for female rats with mammary tissue at reduced risk for developing cancer; parous and tamoxifen treated rats

Months 1-4

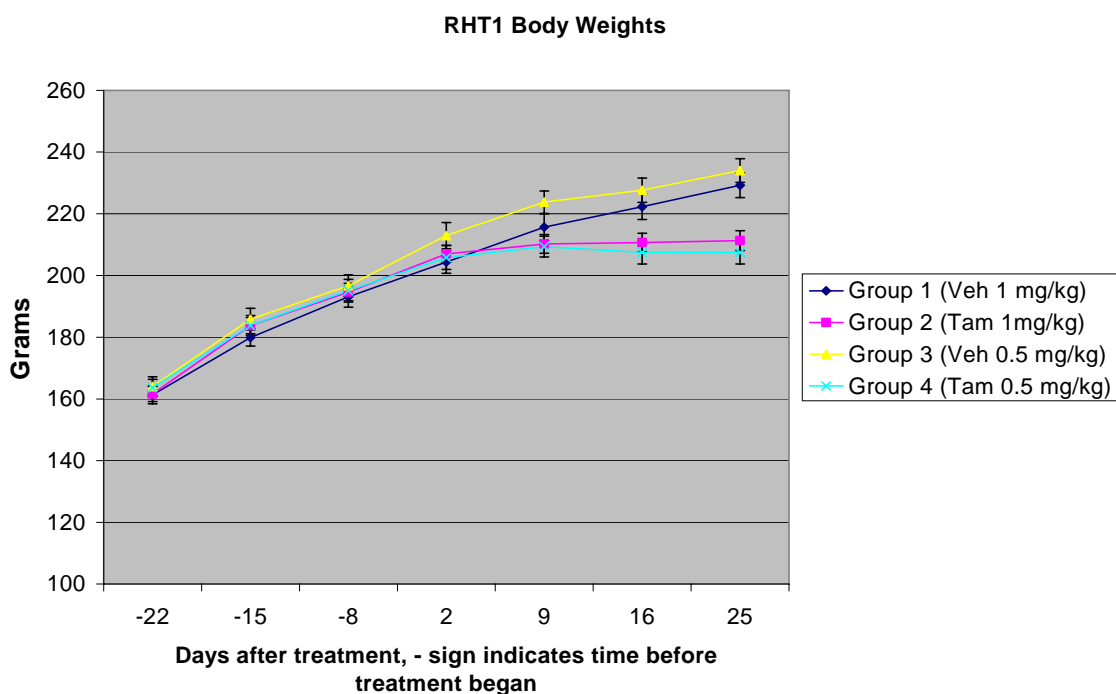
- a) Randomize 90 female Sprague Dawley rats, 50 days of age, into three groups; Group 1, nulliparous control, Group 2, tamoxifen treated and Group 3, parous. Throughout the study, animals will be weighed twice weekly to monitor effects of tamoxifen treatment on body weight gain.
- b) Breed Group 3 rats. After parturition, normalize pups to 8, wean at 21 days of parturition, and allow mammary glands to regress for one month post weaning.

- c) In rats age matched to Groups 1 (nulliparous)& 3 (parous), begin tamoxifen treatment on Group 2. Treat with 0.5 mg tamoxifen/kg body weight, s.c. injection, daily for 4 weeks. Group 1 nulliparous rats will receive daily s.c. injections of vehicle (sesame oil).
- d) At end of 4 week treatment (Gp2) and after 1 month post-weaning (Gp3), all groups will be euthanized.
- e) Two hours prior to euthanasia, inject rats with 50 mg/BrdU i.p. for incorporation into actively proliferating cells.
- f) At time of euthanasia, stage of estrous will be determined by vaginal smear so that inter-animal variation due to estrous can be controlled for across groups. Further, cervical samples will be collected from each rat and used to verify stage of estrous. Based on preliminary studies, it is anticipated that tamoxifen treatment will inhibit cycling.
- g) Inguinal mammary glands, with lymph nodes removed will be flash frozen and stored at -80°C for subsequent ECM isolation. Thoracic glands will be harvested for histological analyses.

Progress Report, Task 1: Tasks 1 a-g were completed and due to mechanical error, all frozen tissue samples were lost on 7/21/06, before any of the tissue was processed for biochemical characterization. Not only were these DOD samples lost, but every experimental tissue, protein and RNA preparations of the entire laboratory were lost. Independent verification of this freezer crisis can be obtained from Jim Gantner, UCHSC Risk System, at 303-315-2732. As a consequence of this catastrophic loss, we are behind on some Statement of Work timeframes. However, due to a tremendous effort on the part of all members of my laboratory, we have made substantial progress towards the objectives outlined in this grant.

Beginning 8/16/06, we repeated Tasks 1 a-g, but omitted Group 3, the parous group. Group 3 was omitted because we felt it was important to narrow the scope of the project during this period of lab-reconstruction. Further, we expanded Group 2 to include two different doses of tamoxifen; 1mg/kg and 0.5 mg/kg bodyweight. We were funded to investigate the effects of 0.5 mg/kg body weight only. However, all of our pilot studies were performed with 1.0 mg/kg tamoxifen per kg body weight. Since our pilot study tissue samples were also lost in the freezer-crisis, we felt it important to repeat the 1.0 mg/kg per body weight arm of the study, so that we had a known effective dose to compare the effects of the 0.5 mg/kgbw dose. To this end, 54 female SD rats at 70 days of age were randomized into 4 groups of 13 and treated for 30 days; Gp1: control rats treated daily with vehicle based on the volume used for 1.0mg/kg tamoxifen dose (approximately 1 ml solvent/rat/day); Gp2: 1.0 mg/kg tamoxifen daily; Gp3: control rats treated with vehicle based on the volume for 0.5 mg/kg and Gp 4: 0.5 mg/kg tamoxifen daily.

Body Weight Data: As can be seen in figure 1, both low and high dose tamoxifen treatment reduced body weight gain. Since difference in bodyweight between groups is a potential confounder, in future experiments the control animals will need to have their daily caloric intake reduced (paired feeding study) so that their body weight gain matches the weight gain of the tamoxifen treated groups.



Task 2-Histological evaluation of mammary gland morphology and proliferative index of mammary epithelial cells.

Months 5-6

- a) To minimize differences in gland morphology due to proximity to the nipple, the same area of gland #4 from each animal will be processed for histological analyses. Three classes of gland morphology will be distinguished; primarily ductal, moderately alveolar (less than 10 ascini per lobule), and alveolar (more than 10 ascini per lobule), using previously published criteria.
- b) Effect of parity and tamoxifen treatment on mammary epithelial cell proliferation will be determined by quantitating BrdU incorporation by immunohistochemical (IHC) methods.

Progress Report, Task 2: All tissue samples have been processed for histological and IHC evaluation. It is apparent from cervical histology, that the tamoxifen treatment was effective at both doses, as epithelial hyperplasia and hormone stimulation are apparent. Mammary gland histology is also consistent with effective drug treatment, although quantification of histology and IHC for BrdU have not been completed.

Task 3-Isolation and characterization of endogenous mammary gland ECM

Months 6-24

- a) For quality control purposes, prior to ECM isolation, mammary gland histology will be performed to ascertain overall gland health. If any animal is found to have

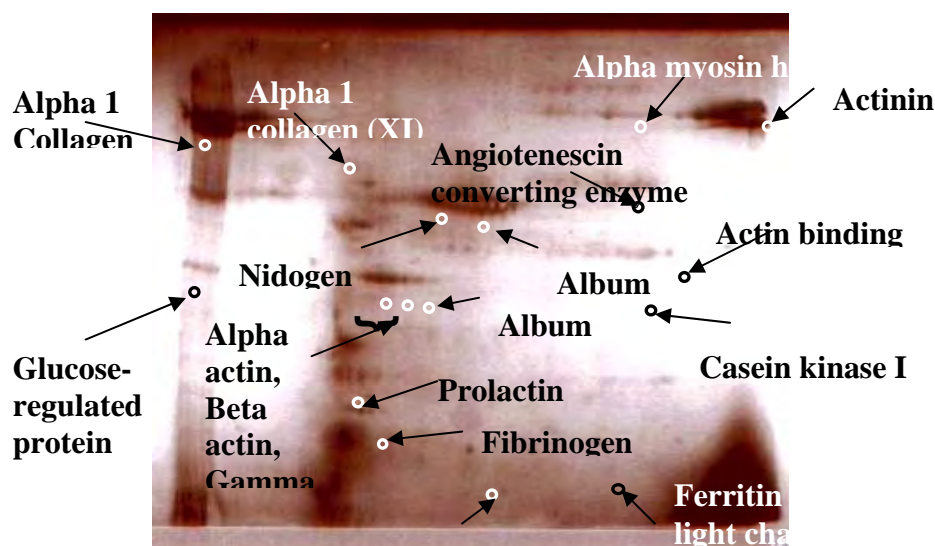
evidence of infection within the mammary gland, the animal will be eliminated from the study. **Progress Report:** Task completed.

- b) Endogenous mammary ECM will be isolated from lymph-node free inguinal mammary glands. **Progress Report:** Task completed.
- c) Perform proteomic analysis of endogenous mammary gland ECM isolated from nulliparous, parous and tamoxifen treated rats using both liquid chromatography mass spectrophotometry (LCMS) and 2-D gel electrophoresis with spot isolation followed by MALTI-TOF-MS. Repeat to control for possible intra-assay variation.

Progress Report, Task 3c

1. 1D Gel Analysis of isolated mammary matrices: mammary ECM has been isolated from vehicle and tamoxifen treated rats. A SYPRO Ruby Stained 1D-Gel (O'Farrell 1975) of the final ECM matrix preparations is shown in Fig 2. Two different lots of Matrigel, the commercially available industry gold standard ECM preparation, are shown as controls. The majority of isolated mammary proteins are above 100kD, consistent with high molecular weight ECM proteins.
2. 2D gel Analysis: we have found, using Matrigel as a control ECM preparation, that resolution of ECM proteins in 2D gels is very poor, primarily because the majority of ECM proteins do not resolve in the first dimension (**Fig 2**). We have found this to be the case even when we prepare our first dimension gel according to the methods of O'Farrell et al (O'Farrell 1975).

Fig 2. 2D-Gel using Matrigel as test ECM. Prominent ECM components of Matrigel, including LN1 were not identified by 2D gel approach.



3. Thus, our focus is now on the use of liquid chromatography mass spec (LCMS) and 1D-Gel band isolation followed by LCMS (**Fig 3**). Protein identification by LCMS of the mammary ECM isolated from treated rats is ongoing.

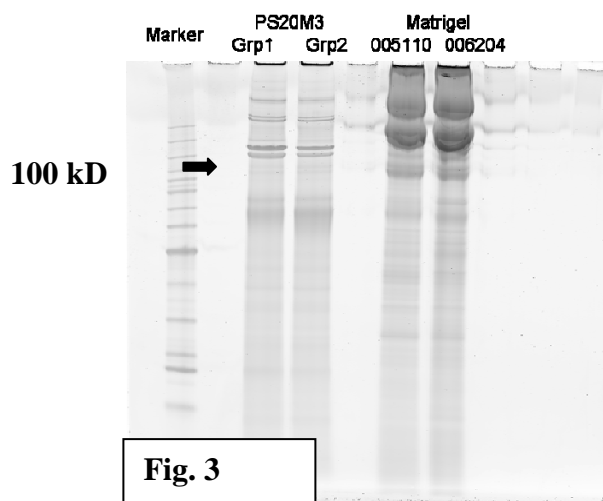


Fig 3. 1D gel of ECM isolated from mammary glands of female rats treated with vehicle (Grp1) and tamoxifen (Grp 2). Two lots of Matrigel are shown as controls.

4. Although not originally proposed in the Schedule of Work, we have elected to try to model the tamoxifen induced changes in the mammary ECM using cell culture models. Our rationale is that determining the proteome of ECM secreted in culture will be technically less demanding than identifying the ECM proteins from ECM isolated from intact mammary glands. This approach is anticipated to provide a more affordable and quicker model system with which to develop the technical advances required for successful ECM proteomics from complex tissues. To this end we have performed two experiments. In the first, we have isolated primary mammary fibroblasts from the vehicle and tamoxifen treated rats and allowed these fibroblasts to lay down ECM in culture. The fibroblasts were then removed and the underlying ECM prepared for MALDI-TOF. In **Fig 4**, the 1D-gel of these ECM preparations is shown. By MALDI-TOF, we have identified a loss of fibronectin (FN) synthesis by the fibroblasts isolated from tamoxifen treated rats. This observation is consistent with our Western blot data demonstrating that FN is reduced in the glands of tamoxifen treated rats compared to vehicle treated (data not shown).

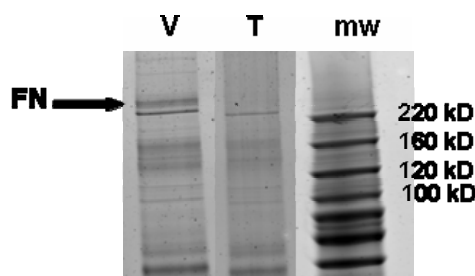


Fig 4. 1D-Gel of ECM laid down in culture by primary mammary fibroblasts isolated from vehicle (V) and tamoxifen (T) treated rats. ECM was solubilized and run on a 1D gel, bands excised, digested and protein identified by MALDI-TOF. Fibronectin (FN) is present in matrix secreted by control (V) fibroblasts and absent from matrix secreted by mammary fibroblasts (T) isolated from tamoxifen treated rats.

5. For our second in vitro model, primary mammary fibroblasts were treated for 10 days in culture with 1 μ m 4-OH Tamoxifen, with media changed and fresh tamoxifen added every two days. The ECM laid down in culture was solubilized and run on a 1D gel, as described above. In **Fig 5**, replica samples are shown with V depicting vehicle treated cells and T depicting tamoxifen treated cells. Differences in protein banding between conditions are not apparent. However, when these samples were analyzed using a quantitative proteomics approach (iTRAQ), differences in ECM were apparent (see point 5 below).

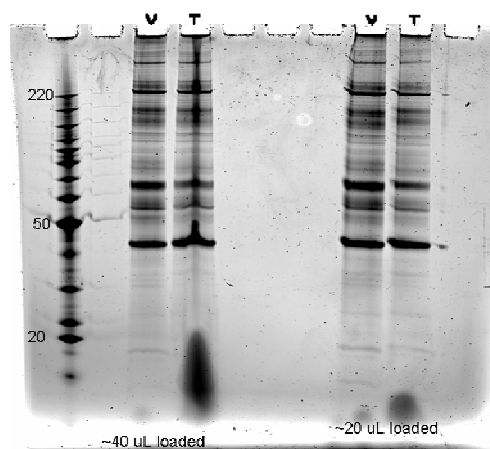


Fig 5. 1D-Gel of ECM laid down in culture by primary mammary fibroblasts treated with vehicle (V) or tamoxifen (T) in culture for 10 days.

6. The ECM proteins isolated from V treated fibroblasts were post labeled using an isotopically labeled reporter tag (isobaric tag 1) that labels primary amines (peptide N-termini and lysine residues). The ECM isolated from the T treated fibroblasts were similarly labeled with a second reporter tag (isobaric tag 2) that has a reporter that differs in molecular weight from tag 1. This protein labeling strategy allows for both qualitative (specific identities of peptides) and quantitative (relative expression of peptides between samples) measurements (Ross, Huang et al. 2004). Using this approach, differences in fibronectin (**Fig 6**), collagen α 1 (**Fig 7**) and vitronectin (**Fig 8**) levels have been observed between V and T treated fibroblasts. The fibronectin data is particularly noteworthy, as a decrease in fibronectin has now been observed with both in vitro and in vivo tamoxifen treatment.

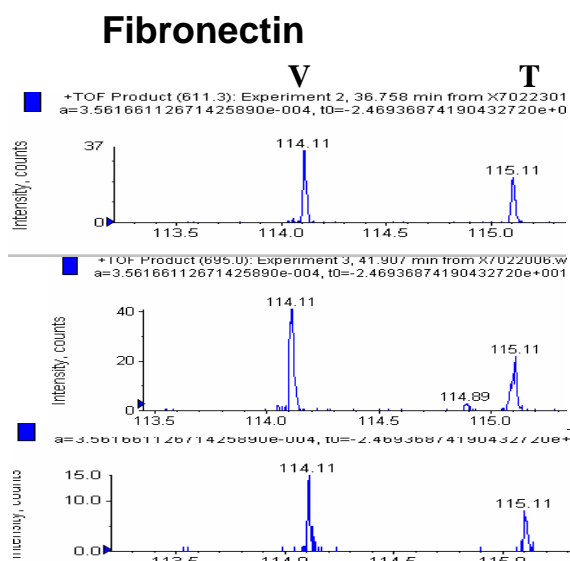


Fig 6. ECM preparations isolated from V treated fibroblasts were labeled with a reporter tag of 114.11 kD and ECM isolated from T treated fibroblasts were labeled with a reporter tag of 115.11 kD. Three separate FN fragments were evaluated for difference in abundance between V and T treatment. Tamoxifen treatment (T) was associated with a reduction in FN abundance.

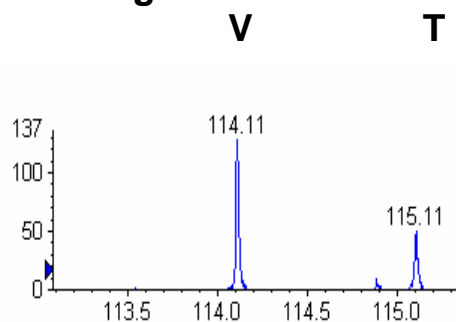
Collagen 1

Fig 7. ECMs isolated and labeled as described in Fig 5 demonstrate reduced levels of collagen 1 were observed with tamoxifen treatment.

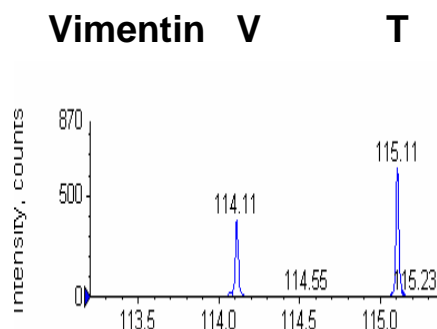
Vimentin

Fig 8. ECMs isolated and labeled as described in Fig 5 demonstrate elevated levels of vimentin were observed with tamoxifen treatment.

Future Directions: 1) Proteomics method development continues with an emphasis on increasing peptide coverage for ECM proteins. 2) ECM proteins identified as being differentially expressed between vehicle and tamoxifen treated cultures will be validated using mammary ECM isolated from tamoxifen and control treated rats.

- d) Repeat steps b-c, using mammary glands from 6 additional animals per group, to control for possible inter-assay variation. **Progress Report:** Not yet begun.
- e) IHC and Western blot validation of proteomics results. To confirm the proteomic results, proteins found by proteomic analyses to be unique or differentially expressed between groups will be evaluated by IHC on tissue sections and by Western blot on mammary gland lysates. For example, if collagen I is found to be less abundant in mammary ECM isolated from tamoxifen treated rats in comparison to nulliparous rats, the relative abundance of collagen I in the mammary stroma will be evaluated by IHC on 5µm mammary gland sections. Further, mammary tissue lysate will be probed by Western blot using Collagen I specific antibodies, as an independent confirmation approach. **Progress Report:** In progress.

Task 4-In vitro assays for evaluating ECM-epithelial cell interactions. Cell lines to be evaluated are non-tumorigenic MCF-10A, tumorigenic but non-metastatic, ER positive MCF-7, and metastatic, ER negative MDA-MB-231 cells.

Year 2, Months 12-24

- a. Prepare mammary ECM, using 6 rats per group per matrix preparation.
- b. In 2D culture, treat cells with endogenous mammary gland matrices at 0, 10, 20 and 40 µg/ml to determine effects of matrices on epithelial cell proliferation and apoptotic indices at 24 and 48 hours post plating, using ³H-thymidine incorporation

assays and apoptosis IHC assays. Experimental conditions to be plated in quadruplicate.

- c. Quantify effects of endogenous mammary on ability of cells to fill in 'wound' in standard scrape assay. Matrices will be tested at 0, 10, 20 and 40 $\mu\text{g/ml}$.
- d. Quantify effects of endogenous mammary matrices on motility and invasion, using ECM preparations as substratum for transwell filter motility and invasion assays. Assays performed in quadruplicate. For motility assays, filters will be coated with 10 $\mu\text{g/ml}$ of the respective matrices. For invasion assays, filters will be coated with 200 $\mu\text{g/ml}$ of the respective matrices.
- e. Use endogenous mammary gland matrix as substratum for epithelial cell organization in 3-D organoid (mammosphere) assays. Each conditions to be tested in quadruplicate. Organoids will be harvested at two time points; 5 and 10 days post plating. Twenty-four hrs prior to harvesting, organoids will be treated with 10 μM BrdU. Organoids will be fixed in 4% paraformaldehyde, embedded, and sectioned into 5 μm sections. Immunohistochemical analyses for markers of cell proliferation, apoptosis, cadherin localization, and apical/basal epithelial cell polarity will be performed.

Progress Report, Task 4: experiments will begin year 2. .

Task 5- Test whether parity and tamoxifen treatment produce a microenvironment suppressive of tumor development.

Year 3, Months 24-36

- a. Thirty six Nu/Nu athymic female mice will be randomized into three groups of 12 mice each; Gp1 Nulliparous, Gp2, tamoxifen treated and Gp3, parous, and treated as described in Aim 1, tasks a, b, and c.
- b. At one month post weaning (Gp3) and after 4 weeks tamoxifen treatment (Gp 2), mice in all groups will be injected into the left and right mammary fat pads of inguinal gland #4 with 2×10^6 MDA-MB-231 cells in 20 μl of media.
- c. Tumor growth will be measured twice weekly using calipers and tumor volume calculated as $\frac{4}{3} \pi R^2 h$. At six weeks post injections, tumors will be excised with a small border of mammary tissue attached, weighed and final volume calculated. Half of the primary tumors will be flash frozen in liquid nitrogen for ECM isolation and the other half fixed and processed for IHC analyses.
- d. The question of whether MDA-MB-231 tumor cells induced a desmoplastic reaction in their surrounding stroma will be evaluated by IHC of tumor/mammary gland border sections. IHC for smooth muscle actin, tenascin, collagen I, endothelial specific marker CD31, and for neutrophil granulocyte infiltration will be evaluated.
- e. To determine whether the microenvironment influences deposition of ECM in the proliferating tumor, ECM will be extracted from the resulting tumors for proteomic analyses.

Progress Report, Task 5

- 1. Using mammary ECM isolated from V and T treated rats for Task 3c, we performed an unscheduled but highly related study. Mammary ECM was mixed with human mammary tumor MDA-MB-231 cells and injected into the 4th

mammary gland of immunocompromised mice. The objective was to determine whether tamoxifen altered the ability of mammary ECM to support tumor growth and metastasis in a xenograft model. Our rationale was based on prior success using this model system to investigate the role of mammary ECM on tumor cell fate (McDaniel 2006). Tumors were harvested at two time points post injection; at 5 days (n=5 mice per group) and 6 weeks (n=15 mice per group). For the 5 day time point, our objective was to determine if tumor cell proliferation was altered by the source of ECM that the tumor cells were mixed with prior to mammary fat pad injection (i.e. V vs T ECM). To this end, 5 mice per group were injected with BrdU 2hrs prior to tumor harvest. Examples of two of these small tumors with brown BrdU positive proliferating cells are shown in **Fig 9**. Quantization of BrdU incorporation is underway.

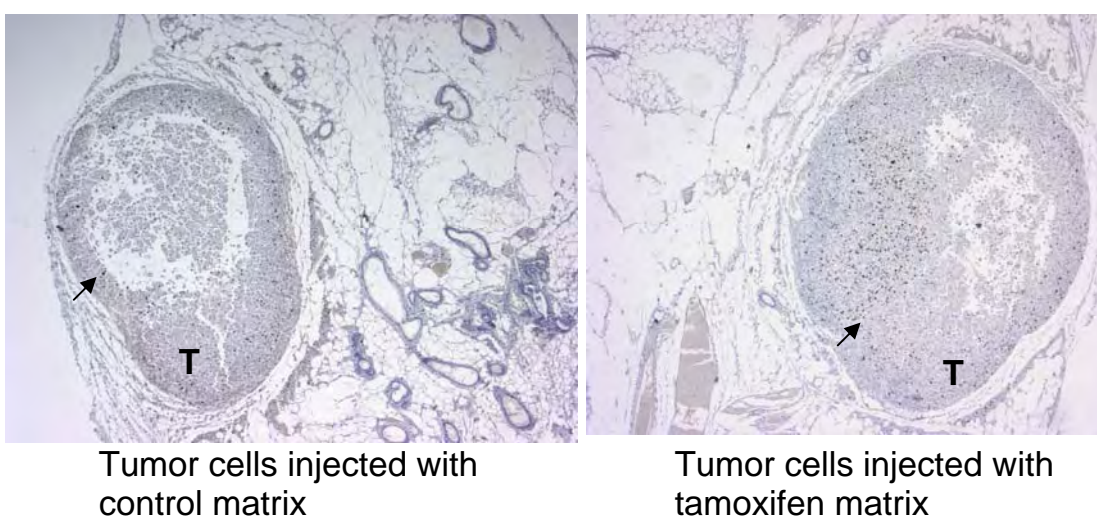


Fig 9. Images of xenograft tumors that developed from MDA-MB-231 cells injected with control ECM (left panel) and tamoxifen ECM (right panel). Arrows depict BrdU positive cells, 40X.

For the 6 week tumor study, tumor size was measured twice weekly using calipers and tumor volume calculated as $\frac{4}{3} \pi R^2 h$. As can be seen in **Fig 10**, tumor growth was delayed in the tamoxifen group in comparison to the control group. These data are consistent with our hypothesis that tamoxifen treatment shifts the mammary ECM environment towards one that suppresses tumor progression and/or supports tumor cell dormancy. We have processed $\frac{1}{2}$ half of each tumor for histological evaluation. The other half of the tumors were quick frozen at -80°C for biochemical evaluation, along with lung, liver and kidney samples from each mouse. Our plan was to utilize these distant tissues to evaluate metastatic events. Unfortunately, all frozen tissues from this study were also lost in the

freezer failure described at the beginning of this report. Histological evaluation of these tumors, as described in Task 5d, will begin year 2.

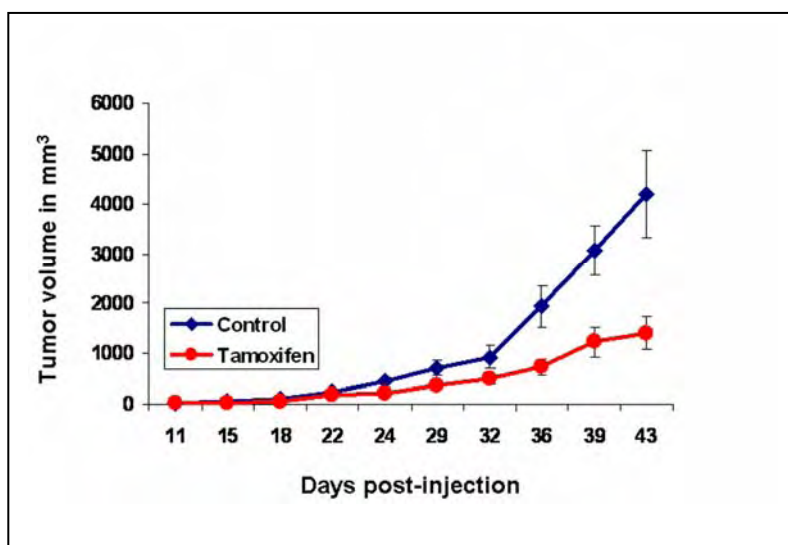


Fig 10. MDA-MB-231 cells were mixed with control mammary ECM or mammary ECM from tamoxifen treated rats, injected into mammary fat pad #4 and tumor growth monitored twice-weekly.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

1. Method development for ECM proteomics.
2. Xenograft model for the study of tumor cell –ECM interactions.
3. Identification of FN as being downregulated in mammary gland of rats treated with tamoxifen.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

1. Abstracts: TAMOXIFEN TREATMENT FUNCTIONALLY ALTERS THE RAT MAMMARY STROMA, INDICATING A ROLE FOR THE MICROENVIRONMENT IN TUMOR SUPPRESSION. R. Hattar, S. McDaniel, P. Schedin, The 25th Congress of the International Association for Breast Cancer Research, Montreal, Canada, Sept 15-18, 2006.
2. Presentations:
 - P. Schedin, Invited Speaker, Gordon Research Conference on Basement Membrane, '*Plasticity of the mammary gland extracellular matrix and breast cancer progression*', Barga, Italy, June 18, 2006.
 - P. Schedin, Visiting Professor, The Cellular and Molecular Basis of Disease, Interdepartmental seminar series, University of New Mexico, '*Plasticity of the mammary gland extracellular matrix and breast cancer progression*'. Albuquerque, New Mexico, Sept 22, 2006.

3. Research Opportunities Applied for: Rhonda Hattar, Predoctoral Training Grant, DOD, 5/2006, entitled 'Using SERMS and Parity as Tools to Investigate the Tumor-Suppressive Microenvironment'. Not funded.

CONCLUSION: In this grant, we hypothesized that it would be possible to gain insight into the role of the microenvironment in tumor cell dormancy by utilizing treatments (or conditions) that target the mammary epithelial cells directly. Our rationale was that because the functional unit of the mammary gland is the epithelial cell plus its ECM, any condition that causes a change in epithelial cell function (such as tamoxifen treatment) would cause a compensatory and reciprocal change in the surrounding stroma (Schedin, Mitrenga et al. 2004; Bissell and Labarge 2005). Our results are consistent with this hypothesis and are already providing insight into the 'tumor suppressive microenvironment'. Our continued work in the area of ECM proteomic development is another independent accomplishment that is likely to benefit the tumor microenvironment field.

Recommendations for changes to future experiments include;

1. The addition of experiments designed to investigate whether the rat mammary stromal cells respond directly to tamoxifen, or whether the changes in ECM in response to tamoxifen treatment are indirect, as a result of changes in epithelium. To this end, we would like to perform IHC studies for ER α and ER β in our fixed mammary tissues obtained in Task 1.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

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- O'Farrell, P. H. (1975). "High resolution two-dimensional electrophoresis of proteins." *J Biol Chem* **250**(10): 4007-21.
- Ross, P. L., Y. N. Huang, et al. (2004). "Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents." *Mol Cell Proteomics* **3**(12): 1154-69.
- Schedin, P., T. Mitrenga, et al. (2004). "Mammary ECM composition and function are altered by reproductive state." *Mol Carcinog* **41**(4): 207-20.

APPENDICES: none provided.

SUPPORTING DATA: none provided.